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The cytogenetic and nuclear changes that occur during tumor progression in the breast have been well documented, but the causes of these alterations are poorly understood. Changes in estrogen receptor status, loss of responsiveness to conventional chemotherapy, gain in metastatic potential, accumulation of PNCs, and differential splicing of CD44 pre-mRNA are examples of changes seen in breast cancer cells during tumor progression. Thus far, a strong connection between the splicing machinery and these subtle, yet significant, changes in gene expression has yet to be documented. Likely candidates are the alternative splicing factors most notably the Polypyrimidine Tract Binding Protein (PTB). PTB is a known repressor of exon definition. We believe that modification of PTB's ability to repress exons, possibly in CD44, to be occurring as breast cells as well as many other cancers. We are attempting to design molecular assays that will display the significance of PTB on the alternative splicing of CD44. We are using a GFP based minigene to define cis elements required for regulation. Thus far, we have been able to demonstrate the versatility of this reporter in living cells and recapitulate PTB-mediated repression on the exon IIIb of FGF-R2, which is another pre-mRNA known to be regulated by PTB and whose regulation is lost during tumor progression.

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Introduction

The broad, long-term goals of this project is to establish a connection between PTB and the loss of regulation of CD44 alternative splicing in breast cancer tumor progression. The specific aims proposed in the original submission of this grant break down essentially into the following: (1) Create minigenes capable of recapitulating endogenous CD44 alternative splicing events, (2) Using this minigene, we will then identify the cis elements required for maintaining regulation, (3) Examine the role, if any, for PTB in regulating the minigene. In order to address this role a minigene has been constructed. We have previously employed the use of a two exon, single intron adenoviral splicing construct capable of being transfected into cells and assayed by RT-PCR. We have had much success studying a parallel system to CD44; the Fibroblast Growth Factor Receptor 2 pre-mRNA splicing. The current summary describes our efforts to design a minigene that will be capable of recapitulating cell-type specific splicing events and well as acting as a readout for PTB activity. This reporter uses GFP fluorescence as its readout rather than RT-PCR. This allows for the ability to view splicing events as they are happening in the living cell. The preliminary experiments using this are very promising and our long-term hope is to put these reporters into mice to eventually determine the physiologic significance of these aberrant splicing events in tumors.

Body

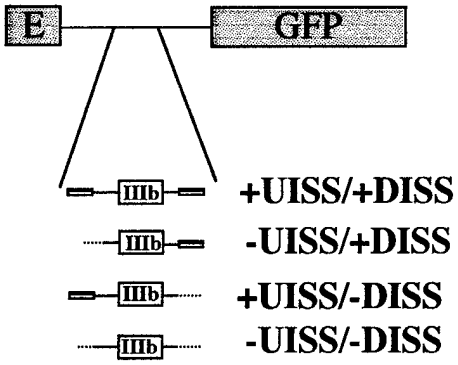
The ultimate goal of this research proposal is to understand the role that PTB has in the regulation of the CD44 alternative splicing and how that role is changed as breast cancer tumors progress into an estrogen independent state. Having an understanding of the general function of PTB will aid in the development of models and the assays to test those models. In the past year we have made tremendous advancement in our general knowledge of PTB function. Our lab, as well as others, has made contributions to understanding how this protein works. These findings as well as my own personal insight are summarized in a recently published minireview in the journal "Molecular & Cellular Biology" (1).

In order to advance our understanding of alternative splicing events in vivo, we have constructed GFP based reporters. We have done this by cloning an intron derived from the adenovirus into the GFP reading frame of the EGFP vector as provided by clonetech. Within the adenoviral intron is a multi-cloning site that allows convenient subcloning of any intronic or exonic sequences (Figure A). We have previously shown that PTB binds to an upstream intronic splicing silencer (UISS) and causes skipping of the Fibroblast Growth Factor Receptor 2 exon IIIb (2). Additionally, we are currently preparing a manuscript outlining a downstream intronic splicing silencer (DISS) that also binds to PTB. Both of these silencers are required to repress exon IIIb. Using this information, we assembled EGFP reporters containing exon IIIb and various deletions of either the

UISS or DISS. These reporters were transfected into DT3 cells, which are a non-metastatic, androgen-dependent rat prostate carcinoma cell line, and fluorescence was measured using fluorescent microscopy. As can be seen, only when both silencers are left intact does IIIb get repressed thus regenerating a productive GFP cDNA. If either of the flanking ISS are deleted, IIIb is included thus causing a deleterious insertion into the GFP cDNA precluding fluorescence (Figure B). This effect is the result of exon IIIb inclusion as verified by RT-PCR (Figure C). When fold change in fluorescence is plotted with fold change in IIIb inclusion, an obvious correlation can be seen. These result, although not pertinent directly to CD44, directly shows that PTB mediated repression can be detected in vivo, thus an in vivo assay for PTB activity is established. As a tool, this construct can be used to address the function of PTB on other alternatively spliced exons including CD44.

We have also shown that two intronic splicing activators (IAS) specifically activate exon IIIb in DT3 cells while they have no function in AT3 cells. We have tested this cell-type specific alternative splicing by designing four more GFP reporters as diagrammed (Figure E). When these constructs are transfected into both cells, we can clearly see that only in the presence of both intact activators (EGFP+ISAR) do you lose fluorescence and this only occurs in the DT3 cells (Figure F). Thus, this reporter can recapitulate cell-type specific alternative splicing patterns.

A.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GFP
IIIb
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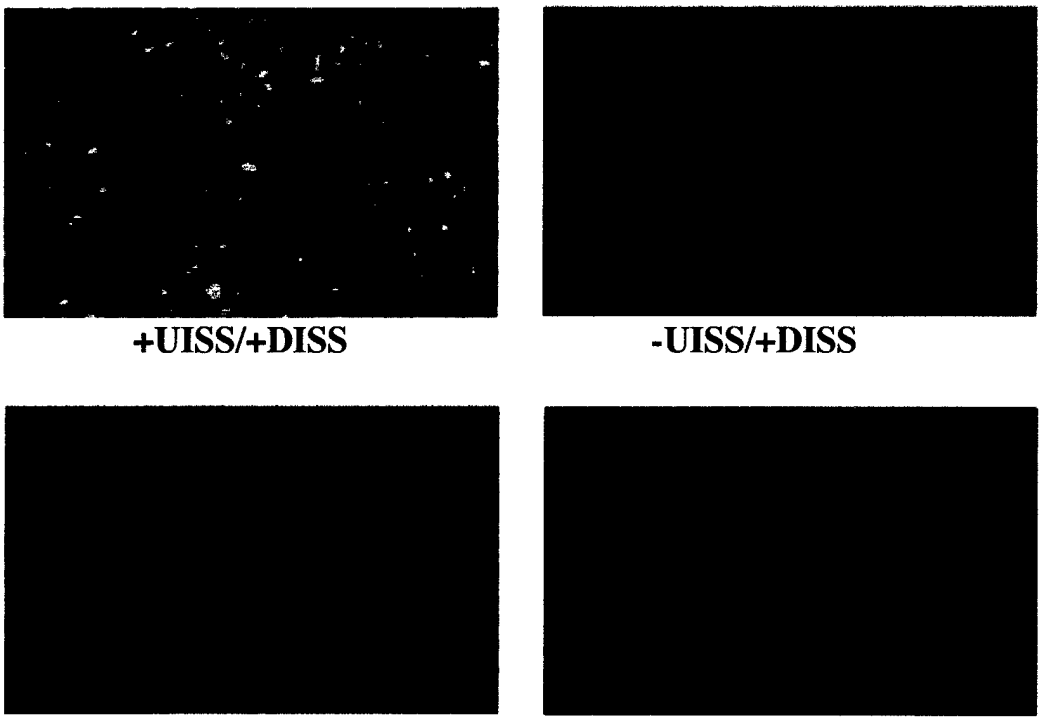
-UISS/-DISS

B.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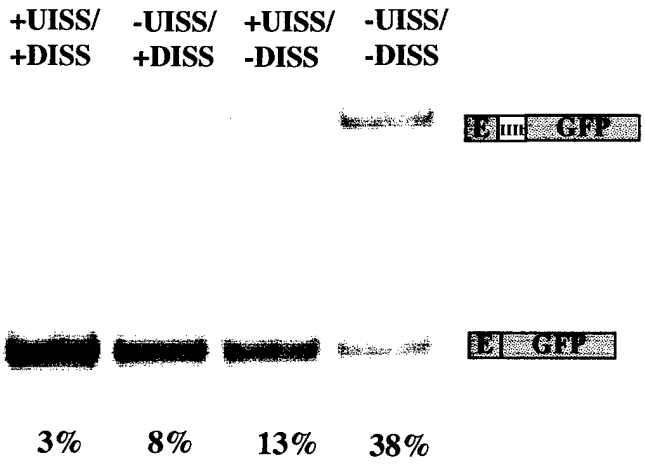
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E GFP
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8%
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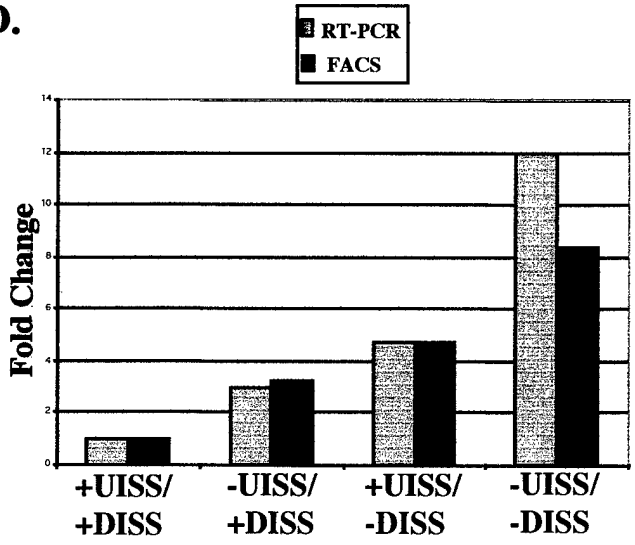
D.


RT-PCR
FACS
Fold Change
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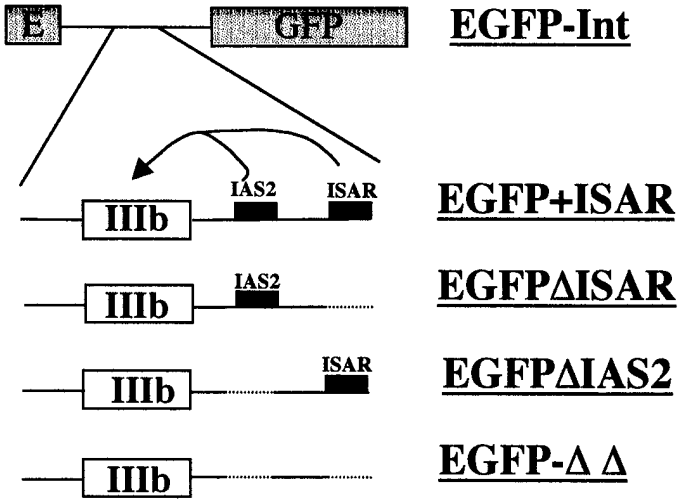
+UISS/ -DISS
-UISS/ -DISS

E.


E
GFP
EGFP-Int
IAS2
ISAR
IIIb
EGFP+ISAR
IIIb
IAS2
EGFPΔISAR
IIIb
ISAR
EGFPΔIAS2
IIIb
EGFP-Δ Δ

F.

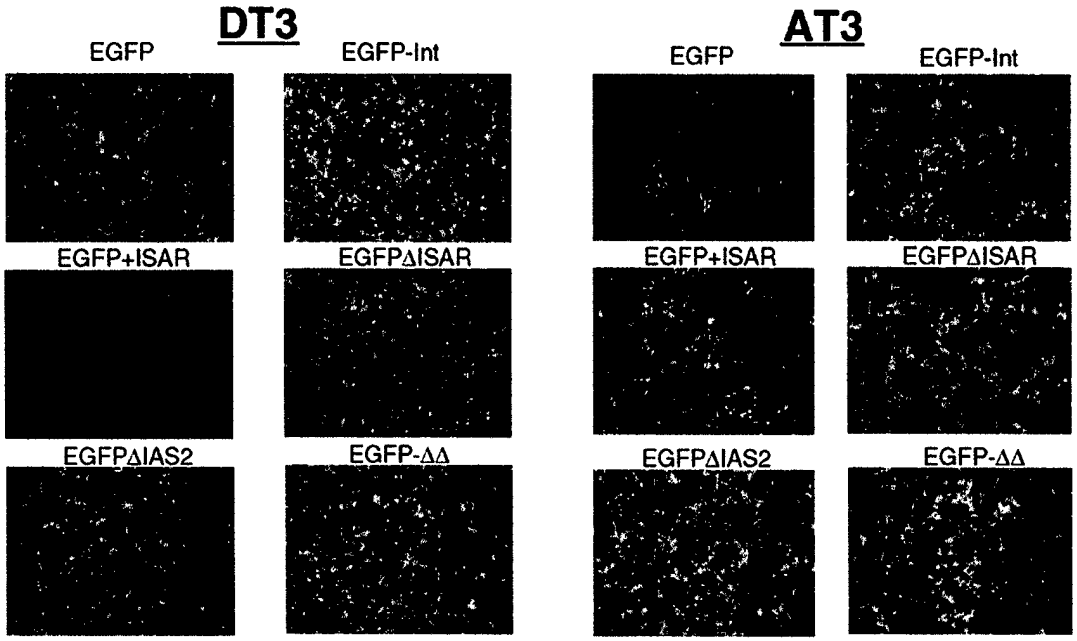

DT3
AT3
EGFP
EGFP-Int
EGFP
EGFP-Int
EGFP+ISAR
EGFPΔISAR
EGFP+ISAR
EGFPΔISAR
EGFPΔIAS2
EGFP-ΔΔ
EGFPΔIAS2
EGFP-ΔΔ

Figure Legends:

A. Into the EGFP cDNA we have cloned an adenoviral intron which splices with near 100% efficiency when transfected into all of the cell lines we have tried (not shown). Into the adenoviral intron we have subcloned the FGF-R2 exon IIIb with flanking intronic sequence. Upstream of IIIb is the Upstream Intronic Splicing Silencer (UISS) and downstream is the Downstream Intronic Splicing Silencer (DISS). Both of these elements bind to PTB (see appendix; Wagner et al. Man in prep). Both elements required to get IIIb skipping. Inclusion of the IIIb exon will disrupt the GFP reading frame thus a loss of fluorescence is seen. Skipping of the internal IIIb exon results in GFP activity.

B. When the four reporters diagrammed in Figure A are transfected stably into DT3 cells (rat prostate androgen-dependent epithelial carcinoma) the GFP fluorescence pattern is seen. Only in the case where both silencers are present do we see high levels of fluorescence.

C. To verify that the effects of fluorescence are in fact due to exon IIIb inclusion, we assayed for exon inclusion using RT-PCR. As can be seen, as silencers are deleted, IIIb inclusion is increased. Percentage inclusion is labeled below each lane.

D. To compare the relative changes in fluorescence as measure by FACS with the relative changes in RT-PCR exon IIIb inclusion we plotted both effects as "fold change versus the ++ construct". As can be seen, there is a statistical correlation between IIIb inclusion and fluorescence reduction.

E. Construction of four EGFP reporters testing the effects of the cell-type specific activators IAS2 and ISAR. A deletion series of each element was made totaling four constructs.

F. The four constructs (as well as EGFP alone and EGFP with the intron alone) were transfected stably into DT3 and AT3 cells. As can be seen when ISAR and IAS are both present, low levels of fluorescence is seen due to IIIb inclusion. This observation is not seen in AT3 cells, which do not recognize ISAR and IAS.

Key Research Accomplishments:

-Designed EGFP minigenes

-Subcloned several cis elements as well as exons into EGFP minigene

-Demonstrated PTB-mediated repression of Exon IIIb

-Demonstrated cell-type specific effects of IAS2 and ISAR

Reportable Outcomes:

-**Manuscript: Wagner EJ, & Garcia-Blanco MA.** 2001 The Polypyrimidine Tract Binding Protein Antagonizes Exon Definition. Mol. Cell. Biol. **21:** 3281-3288 (See appendix)

-**Manuscript: Carstens RP*, Wagner EJ*, & Garcia-Blanco MA.** 2000. An Intronic Splicing Silencer Causes Skipping of the IIIb Exon of Fibroblast Growth Factor Receptor 2 through Involvement of Polypyrimidine Tract Binding Protein. Mol. Cell Bio. **20**: 7388-7400 **(*co-first authors).** Please Note: This work was performed just prior to funding period but was Published during funding period. The work presented in this paper Is consistent with numerous aspects of the proposed research. (See appendix)

-Presentation: Oral Presentation at the Annual RNA Society Meeting held in Banff Alberta. Title of Abstract: Cell-type specific Inclusion of FGF-R2 exon IIIb is mediated by a central switch: the intronic control element. (See appendix)

-Presentation: Submitted Oral Presentation to the Cold Spring Harbor RNA processing Meeting in August. Title of Abstract: Fluorescent Reporters to Recapitulate in vivo Changes in FGF-R2 Alternative Splicing. (See appendix)

-**Awards-** Student Researcher of the Year Award from the American Cancer Society; given to one Duke University student who is performing research in an ACS funded lab

Conclusions:

The results obtained thus far, although do not yet provide direct insight on the CD44 alternative splicing phenomenon will supply molecular biologist with an additional tool for studying splicing events. The EGFP reporters have been demonstrated to faithfully recapitulate cell-type specific alternative splicing of FGF-R2. Additionally, these reporters provide a direct readout for PTB-mediated exon silencing. Thus, it appears that the development of the tools for more general purposes, which may include CD44 regulation, is possible. To answer the question of "so-what?" is simple. Little is known about the physiological significance of aberrant alternative splicing of CD44 or FGF-R2. A possible long-term use for these fluorescent reporters is to re-introduce them into animals in the form of in situ tumors. Fluorescent patterns of metastasized and non-metastasized cells can be measured and is directly reflective of alternative splicing events. Thus, we may gain insight on the cause and effect relation between metastasis and the loss of regulation of alternative splicing.

References

All of the references within this report are attached in the appendices

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MINIREVIEW

Polypyrimidine Tract Binding Protein Antagonizes Exon Definition

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The removal of introns from mRNA precursors (pre-mRNAs) involves two relatively straightforward chemical reactions. The recognition of intron-exon boundaries, the splice sites, however, requires the integration of information provided by many *cis*-acting elements and a complex splicing machinery (64). The *cis*-acting elements that define the borders between exons and introns are quite diverse and yet are recognized efficiently by the splicing machinery. This machinery is composed of general splicing factors (GSFs), which make up the spliceosome and its associated proteins, and of regulatory factors. The same machinery must also make cell-type-specific choices in cases in which pre-mRNAs are alternatively spliced. This is a monumental task given that it is estimated that transcripts from 30% of all genes in humans are alternatively spliced (http://devnull.lbl.gov:8888/alt).

The spliceosome, like many macromolecular machines, is not preassembled as an active enzyme but rather assembles on the substrate. The substrate, a functional pre-mRNA, is thought to first interact with U1 snRNP, hnRNP proteins, and SR proteins (5, 34). The interaction is determined by RNA-RNA base pairing between the 5′ end of U1 snRNA and the consensus sequence at the 5′ splice site and by interactions mediated by protein factors (34). The protein factors U2AF and SF1 also recognize the polypyrimidine tract, the branch point, and the 3′ splice site, thus bridging the two groups that subsequently will be involved in the first transesterification reaction. This leads to the formation of the commitment complex (CC). The interaction between the protein factors and the 3′ splice site of an internal exon is enhanced by the binding of U1 snRNP at the downstream 5′ splice site (35, 59). This interaction is the basis for exon definition, an idea discussed in greater detail below. The CC and the U2 snRNP interact to yield the prespliceosome, and the branch point sequence is recognized again, albeit differently in this complex. The prespliceosome interacts with a preformed U5-U4-U6 tri-snRNP to form the immature spliceosome, which then undergoes rearrangements that result in the formation of a fully competent enzyme. This interplay of multiple protein factors and RNA components sets the stage for numerous opportunities for and targets of regulation.

The complexity of constitutive and alternative splice site recognition suggests multiple layers of regulation, with each layer the result of combinatorial arrays of elements and factors (38, 48, 64). The first layer is direct sequence recognition that likely occurs early in the formation of the spliceosome. U1 snRNA can read the sequence at the 5′ splice site, and protein factors SF1, U2AF^{65}, and U2AF^{35} recognize the branch point, the polypyrimidine tract, and the 3′ splice site, respectively (3, 5, 45, 61, 75). These and other GSFs interact with each other and can act as molecular rulers sensing the relative locations of the *cis*-acting elements. Positional and distance information provides a second layer of discrimination that overlies the detection of individual binding sites. This type of information is transmitted via protein-protein interactions in the definition of exons (2). Another example of this type of distance detection is seen in the α-tropomyosin pre-mRNA (63), where the close proximity of the 5′ splice site of exon 2 to the branch point upstream of exon 3 precludes the inclusion of both exons into the mRNA. Modulation of splice site strength by proteins of the SR family provides yet another layer of regulation (33, 66, 71). SR proteins play roles in constitutive splicing and can be considered GSFs; however, in some instances SR proteins have important roles in alternative splicing. These proteins can be recruited directly to the RNA by enhancer elements in exons or introns or indirectly by interactions with other GSFs (36, 43, 58, 67). hnRNP proteins, some of which bind all pre-mRNAs, can also influence splice site choices, possibly by counteracting SR proteins (6). hnRNP A1 and polypyrimidine tract binding protein (PTB), two proteins classified as hnRNP proteins, repress certain splicing events and thereby provide a layer of negative regulation. Very precise regulation is provided by the existence of cell-type-specific factors; several of these have been described in *Drosophila melanogaster* (38). The integration of the information in these regulatory layers leads to splice site choice.

Negative regulation of exon inclusion is emerging as a critical layer in splice site choice. Fairbrother and Chasin considered why certain exons are selected, while others, which seem perfectly competent, are ignored (21). These authors suggest that many, and possibly all, exons are under a global repressive influence mediated by many intronic sequences (21). Thus, splice site utilization can be described as a function of both splice site strength and the intensity of the repressive field within a specific region of a pre-mRNA. This global repressive influence can also contribute to the outcome of regulated alternative splicing events, setting the stage for cell-type-specific derepression of exons (1, 8, 12, 46–49, 72, 73). In mammalian cells PTB has been identified as a key splicing repressor. In this review we critically evaluate the role of PTB in exon silencing

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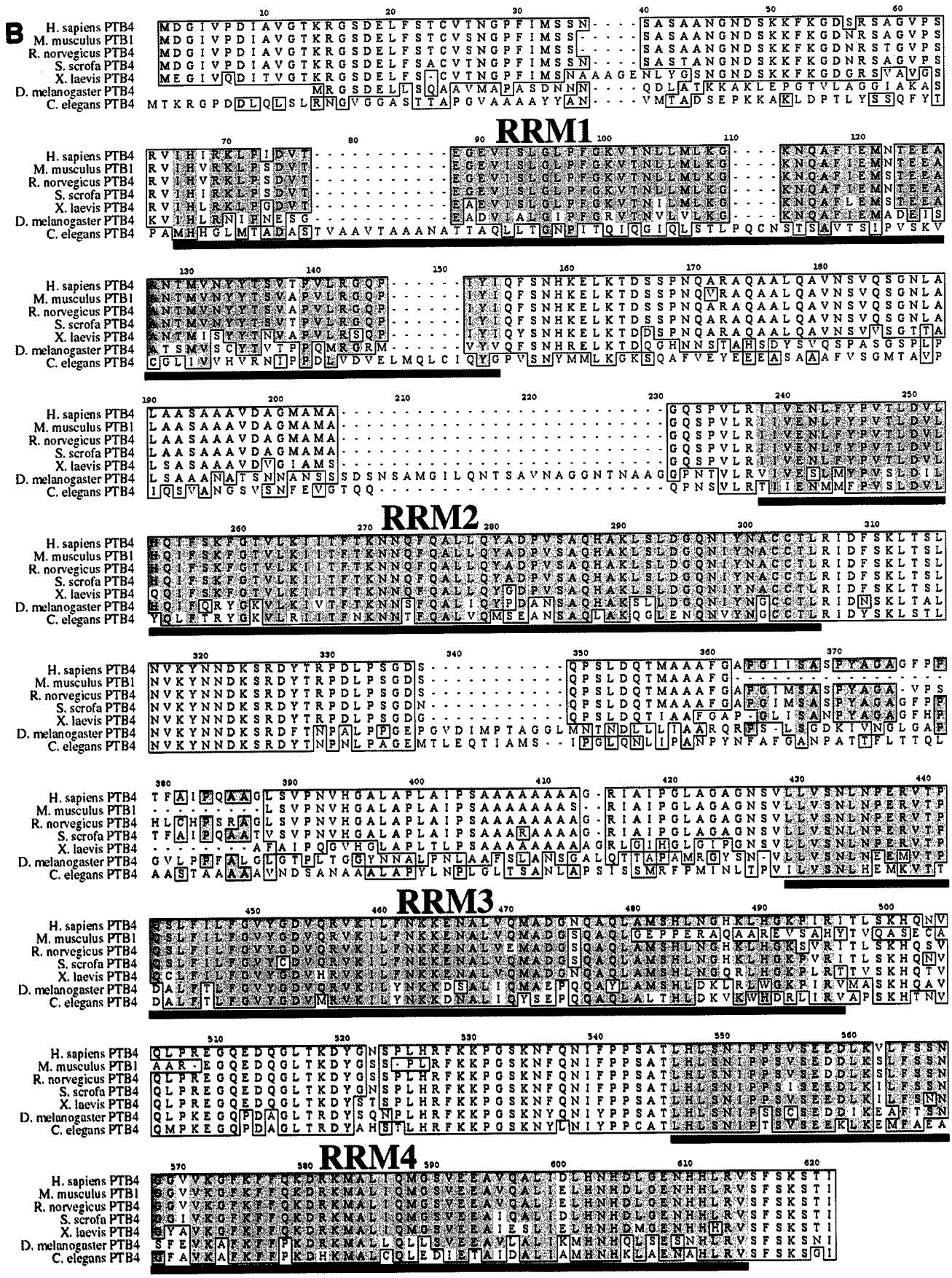

FIG. 1—*Continued.*

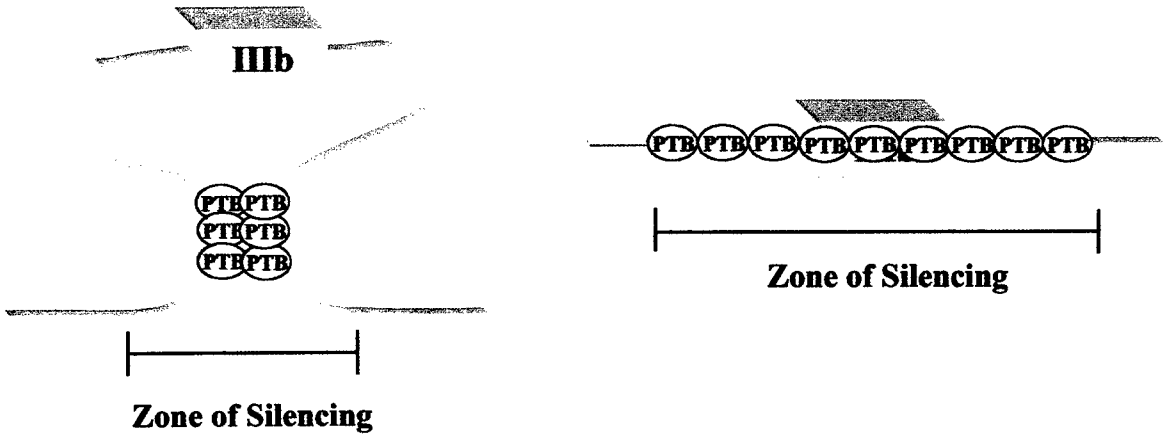

FIG. 3. Two potential mechanisms to define a zone of silencing. The left model predicts that PTB-PTB interactions between binding sites flanking an exon sequesters an exon, thus precluding the definition of this exon. The model on the right suggests that PTB can oligomerize across an exon, resulting in the coating of the exon, which will also antagonize its definition. The model on the right could also explain the silencing of exons that have a PTB site only on one flank; PTB could multimerize, covering a region of the RNA determined by interactions with other factors.

splicing pathway leading to exon 5 inclusion (30) (Fig. 2). PTB would repress the use of the zero-length exon predicted in recursive splicing, thus freeing U1 snRNP to activate polyadenylation. Overexpression of PTB would therefore increase the likelihood of this event. In this case, as in those mentioned above, PTB can be best thought of as an antagonist of exon definition (Fig. 2).

Although PTB has the ability to interfere with exon definition, it seems likely that the presence of PTB binding sites is not sufficient to silence otherwise robust exons (25, 26, 37, 55). This may be the case for exon 3 of the rat α-tropomyosin pre-mRNA, which is silenced in smooth muscle cells but not in many other tissues. Although silencing of exon 3 requires PTB binding sites, it cannot be mediated by PTB alone, given that this protein is found in cells where exon 3 is included. Regulated weak exons require other silencer sequences or weak splice sites in order to achieve repression of exon inclusion. This is clearly the case with the IIIb exon of FGF-R2 in which, in addition to elements that bind PTB, silencing requires a weak polypyrimidine tract and an exonic silencer that interacts with hnRNP A1 (8, 16, 19). PTB also appears to be a component of a multiprotein complex that assembles on regulatory elements in c-src and β-tropomyosin pre-mRNAs (10, 12, 29, 64). Together, these data suggest that PTB acts in concert with corepressors to mediate exon silencing. Given its ubiquitous distribution in cell lines and tissues, it is likely that PTB provides global repression of weak exons. PTB may play a dual role by not only discriminating between splice sites and pseudo-splice sites during constitutive splicing but also setting the stage for cell-type-specific selection of regulated exons during alternative splicing.

MECHANISMS OF PTB REPRESSION

PTB binding sites sometimes overlap binding sites for $U2AF^{65}$, and simple competition could account for the inhibitory action of PTB (37, 62). A well-characterized example of a potential competition model is the repression of the 24-nucleotide exon of the $GABA_A\gamma2$ pre-mRNA. In this case, there are four silencer sequences that bind PTB and act in concert to inhibit the recognition of this exon. Three of these PTB binding sites surround the branch point, with one sitting within the associated polypyrimidine tract (Fig. 2). In this particular example, PTB may be acting in a manner similar to the *D. melanogaster* female-specific splicing factor SXL on TRA pre-mRNA (28, 68). SXL has but one site on the TRA pre-mRNA and excludes access to $U2AF^{65}$ when bound, thus repressing the use of the non-sex-type-specific exon. In most other instances, PTB binding sites do not directly overlap the binding sites of GSFs (9, 10, 12, 25, 26, 55, 65). In fact, it has recently been demonstrated for the SM exon in α-actinin that the critical sites do not overlap with the branch point and polypyrimidine tract of intron 3 (J. Southby and C. Smith, personal communication). Therefore, in this case and many others, PTB-mediated exon silencing is clearly not caused by direct competition with GSFs (see discussion about SXL below). Although these observations suggest that PTB may act by two distinct mechanisms, we argue below for a more parsimonious explanation.

The majority of exons silenced by PTB are flanked by PTB binding sites on both adjacent introns (Fig. 2). Given that PTB can multimerize, it has been postulated that PTB proteins can interact across the exon (12, 53, 56) (Fig. 3). Chou et al. (12) have shown evidence for such an interaction in vitro, demonstrating that mutations in the upstream binding site affected binding of PTB to the downstream site and vice versa. A very similar interaction was proposed for hnRNP A1 proteins binding on either side of a regulated exon of the hnRNP A1 pre-mRNA (4). In that case the effect was postulated to be activation of the downstream exon by approximation. The idea that PTB can interact across exons fits well with the evidence that PTB is an antagonist of exon definition. The PTB sites flanking silenced exons could define a repressive zone within a pre-mRNA. Binding on both flanks is most reminiscent of the postulated mechanism for SXL autoregulation (31, 32). The SXL protein binds in numerous positions flanking the male-specific exon 3 in its own pre-mRNA and prevents its inclusion. SXL interacts with the *Drosophila* U1A/U2B″ homolog, a component of both U1 and U2 snRNPs, and is likely to interfere

exon IIIb is mediated by at least two cell-type-specific *cis* elements and others that appear to be activated in all cells (18). Two of the cell-type-specific elements can form a predicted RNA secondary structure in which one stem would be located between seven consensus PTB binding sites in a downstream intronic silencer sequence (17) (Fig. 4). Formation of this structure might be expected to interfere with PTB binding and exon silencing.

SUMMARY

PTB appears to be a global repressor of weak or regulated exons. We propose here that PTB multimerization sequesters these exons to prevent exon definition. This is likely critical not only to prevent inclusion of pseudo-exons but also to set up cell-type-specific exon definition. What remains unclear about PTB can probably be broken down into two basic questions. First, what is the precise mechanism of repression? Second, how is this mechanism circumvented? Most of the research to resolve the first question has focused primarily on identifying instances of PTB repression but has done little to understand how that repression is achieved. Recently, both in vivo and in vitro assays for PTB repression have been developed (8, 65); thus, a detailed structure-function analysis can be done. Information from this approach may address mechanistic questions such as if PTB multimerization is required for repression or if there are PTB cofactors. Understanding how this repression is lifted will probably be a more complicated issue. Overwhelming PTB may occur by numerous mechanisms, such as strengthening weak splice sites via activators such as TIA-1 (18), causing the enhancement of inclusion via a tissue-specific expression of antagonizing RNA-binding proteins, or simply by modulating the expression of a PTB cofactor.

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An Intronic Splicing Silencer Causes Skipping of the IIIb Exon of Fibroblast Growth Factor Receptor 2 through Involvement of Polypyrimidine Tract Binding Protein

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Alternative splicing of fibroblast growth factor receptor 2 (FGF-R2) transcripts involves the mutually exclusive usage of exons IIIb and IIIc to produce two different receptor isoforms. Appropriate splicing of exon IIIb in rat prostate cancer DT3 cells requires a previously described *cis* element (ISAR, for "intronic splicing activator and repressor") which represses the splicing of exon IIIc and activates the splicing of exon IIIb. This element is nonfunctional in rat prostate AT3 cells, which repress exon IIIb inclusion and splice to exon IIIc. We have now identified an intronic element upstream of exon IIIb that causes repression of exon IIIb splicing. Deletion of this element abrogates the requirement for ISAR in order for exon IIIb to be spliced in DT3 cells and causes inappropriate inclusion of exon IIIb in AT3 cells. This element consists of two intronic splicing silencer (ISS) sequences, ISS1 and ISS2. The ISS1 sequence is pyrimidine rich, and in vitro cross-linking studies demonstrate binding of polypyrimidine tract binding protein (PTB) to this element. Competition studies demonstrate that mutations within ISS1 that abolish PTB binding in vitro alleviate splicing repression in vivo. Cotransfection of a PTB-1 expression vector with a minigene containing exon IIIb and the intronic splicing silencer element demonstrate PTB-mediated repression of exon IIIb splicing. Furthermore, all described PTB isoforms were equally capable of mediating this effect. Our results support a model of splicing regulation in which exon IIIc splicing does not represent a default splicing pathway but rather one in which active repression of exon IIIb splicing occurs in both cells and in which DT3 cells are able to overcome this repression in order to splice exon IIIb.

Alternative splicing represents a commonly used pathway through which different gene products can be produced from a single gene. In many cases of alternative splicing, the splicing pattern is tightly regulated such that distinct cell types differentially splice a given pre-mRNA to produce different protein isoforms. *cis*-acting elements which, when located in an exon, can act either to activate or repress splicing have been identified and characterized (13, 19, 33, 36, 51, 55, 56, 60–62, 66). In addition, intron sequences which either activate or block splicing of adjacent exons have been described (reference 6 and references therein; 9, 11, 45, 53). In more than one case, it has been demonstrated that both positive and negative regulatory *cis* elements are present within a single alternatively spliced transcript (6, 8, 11, 45). Pre-mRNA splicing is known to take place in the spliceosome, a large multicomponent enzymatic machine which consists of the U1, U2, U4/6, and U5 small nuclear RNAs (snRNAs) along with associated small nuclear ribonucleoproteins (snRNPs) and non-snRNP proteins (3, 59). The mechanisms which operate to direct this spliceosomal apparatus to yield alternatively spliced RNAs have been poorly defined in mammalian systems to date (59). Well-described examples of cell-specific factors in *Drosophila* which can act positively or negatively to alter the splicing of specific exons have been proposed to be models for alternative splicing in mammals (reviewed in reference 40). Nonetheless, such purely cell-specific factors have not been identified in mammals, and ongoing debate centers around the question whether analogous cell-specific alternative splicing factors will be found to modulate the processing of mammalian gene transcripts. It has been proposed that mammals have adapted mechanisms which rely on relative differences in the levels of multiple factors, which regulate pre-mRNA splicing in a combinatorial manner (28, 45).

A number of nonspliceosomal proteins that are not tissue restricted are capable of altering the splicing of a number of different pre-mRNA substrates. Several SR protein family members bind exonic enhancer sequences to increase the inclusion of the corresponding exon (33, 35, 36, 51, 54, 55). In addition, SR proteins have differential effects on splice site selection. ASF/SF2, for example, promotes the use of a proximal 5′ splice site upstream of a defined 3′ splice site, an effect which can be counteracted by heterogeneous nuclear RNP A1 (hnRNPA1) (4, 17, 20, 39). Two other hnRNPs, hnRNP F and hnRNP H, are components of a complex that forms on a neural cell-specific intronic enhancer element, resulting in the increased splicing of the N1 exon of c-*src* (11, 43). KH-type splicing regulatory protein (KSRP) is a component of this complex, although its expression, like that of hnRNP F and hnRNP H, is not neural cell specific (44). In contrast to its role in activating the splicing of the N1 exon, hnRNP H binds to an exonic splicing silencer in β-tropomyosin and has been proposed to cause the exclusion of exon 7 in nonmuscle cells (9).

Polypyrimidine tract binding protein (PTB) was originally purified based on its ability to bind to an adenovirus polypyrimidine tract and was subsequently also described as hnRNP-I (2, 18, 21, 22, 47). A role for PTB in alternative splicing was first proposed by Mulligan et al. studying the alternative splic-

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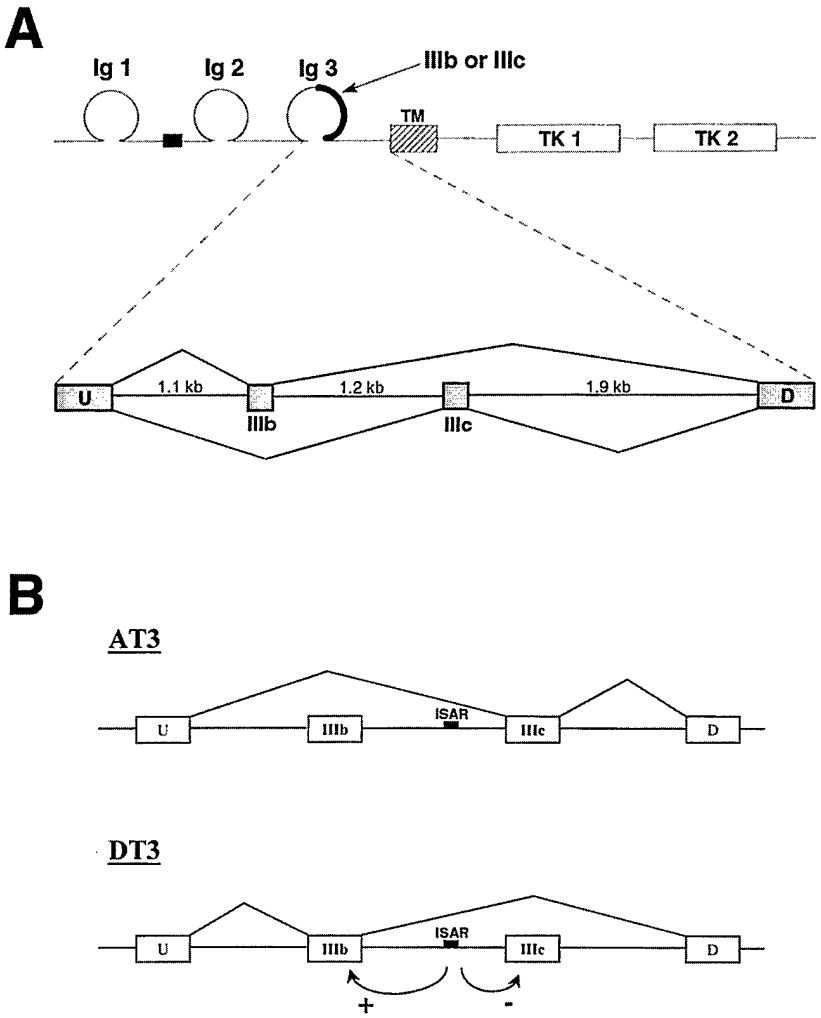
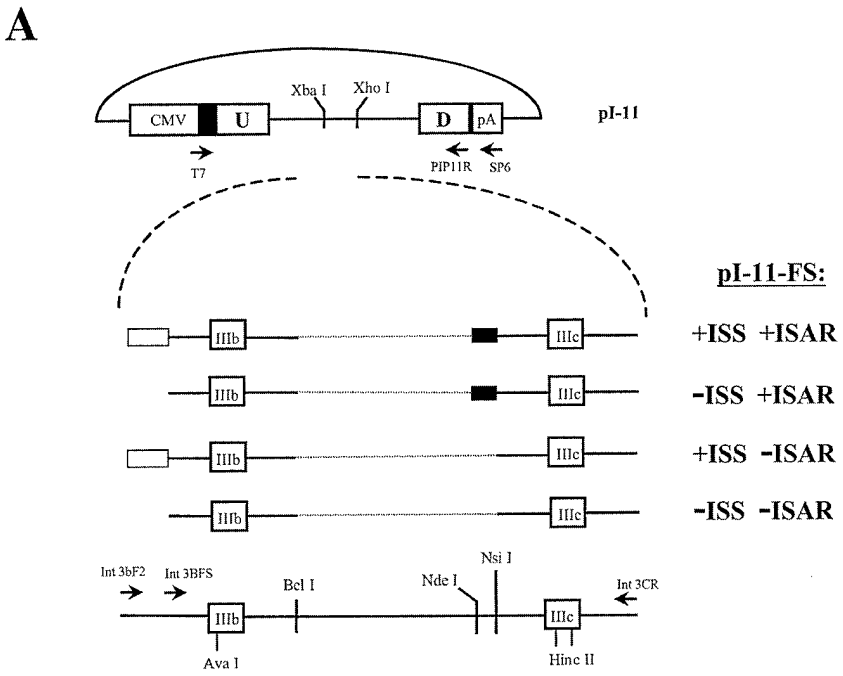


FIG. 1. Schematic representation of the alternative splice variants of FGF-R2. (A) Diagram showing the relative positions of exons IIIb and IIIc in the context of a protein domain map of FGF-R2. Abbreviations denote the immunoglobulin-like domains (Ig 1 to Ig 3), transmembrane domain (TM), and both intracellular tyrosine kinase domains (TK). (B) The AT3 and DT3 cells splice to the mutually exclusive exons IIIc and IIIb, respectively. In DT3 cells, the previously described *cis* element, ISAR, functions both to activate IIIb inclusion and to repress IIIc inclusion. In AT3 cells, there has been no detected function for ISAR, and so IIIc is included regardless of the presence of ISAR.

RC-ΔEX-ISS1, pDP-RC-ΔEX-ISS1mt, and pDP-RC-ΔEX-Globin were used for the synthesis of hot probes ISS1, ISS1mt, and Globin, respectively. These constructs were created by cloning annealed oligonucleotides F-WT and R-WT (for ISS1), F-MT and R-MT (for ISS1mt), and F-BG and R-BG (for Globin) into the *Eco*RI and *Spe*I sites of pDP-RC-ΔEX. pDP-RC-ΔEX was created using pDP19 (Ambion) but replacing the entire polylinker between the *Eco*RI and *Hin*dIII sites of this vector with the sequence 5′-tagaactagtgcggccgcatatcatcgatgctcgag-3′. Oligonucleotide sequences were as follows: F-WT, 5′-aattctcattgtgatctcc tccctccca cagctctttaggtgtaa-3′; R-WT, 5′-ctagttacacctaaagagct gtgggagggaggagatcacaatgag-3′; F-MT, 5′-aattctcattgtgatggagaaa ggaccacagctctttaggtgtaa-3′; R-MT, 5′-ctagtt acacctaaagag ctgtggtcctttctccatcacaatgaG-3′; F-BG, 5′-aattggagaccaatagaaa ctgg gcatgtggagacataggtgtaa-3′; R-BG: 5′-ctagttacacctatgtctcc acatgcccagtttctattggtct cc-3′.

PCR amplification and RT-PCR assay of transfected minigenes. PCR from DNA templates for plasmid construction was done using standard reaction conditions as described previously (6). RNA for reverse transcription-PCR (RT-PCR) assays was isolated using the method of Chomczynski and Sacchi (10). RT-PCR using T7 and SP6 primers to analyze pooled stable transfections was performed as described previously (6). When RT-PCR was used to assay results from transient cotransfections, the RNAs were first treated with RQ1 DNase (Promega), as specified by the manufacturer, to eliminate background from residual plasmid DNA templates. Also, in this case, T7 was used with primer PIP11R, which corresponds to the sequence at the 3′ end of the second exon of pI-11: 5′-ccggactagtaagcttaggctcttggcgtt-3′. In all amplification reactions, a water control and a mock RT control were included, which resulted in no PCR product in all experiments. PCR products were either loaded directly onto 5% nondenaturing polyacrylamide gels or, when necessary, added to restriction endonuclease digestions with either *Ava*I or *Hin*cII (New England Biolabs). We always observed complete digestion when using this method. Aliquots representing equal amounts of each PCR mixture with undigested and digested PCR products were loaded onto 5% polyacrylamide gels. The gels were electrophoresed at 100 V for 3 to 4 h, dried, and exposed to Amersham Hyperfilm-MP or Molecular Dynamics phosphorimager screens. Analysis was performed with a Molecular Dynamics PhosphorImager. Quantifications were performed as follows. For DT3 and AT3 cells (see Fig. 2B and C), we quantified the percentages of the spliced products that contained both exons IIIb and IIIc (U-IIIb-IIIc-D), those that contained either exon IIIb or exon IIIc (U-IIIb/IIIc-D), and those that skipped exons IIIb and IIIc and spliced the adenovirus exons together (U-D) by adding the values for the bands representing these products from the undigested RT-PCR lanes (corrected for molar equivalents) and representing each as a percentage of the total (where U and D are the 5′ and 3′ exons, respectively, of pI-11). In addition, data were quantified to assess the percentage of the single-inclusion product that contained IIIb. This was done by using the quantification of the band at 380 bp, which remained following *Hin*cII digestion (U-IIIb-D), as the numerator. The denominator consisted of U-IIIb-D and the 377-bp band that remained following *Ava*I digestion (U-IIIc-D). Quantification of experiments using minigenes with only one internal exon (exon IIIb) (see Fig. 3B and C) was determined as the quantification of the 380-bp band (U-IIIb-D) divided by the sum of the same band and the exon IIIb-skipped 232-bp band. Quantifications for Fig. 6C were done by dividing the amount of IIIb inclusion (as calculated for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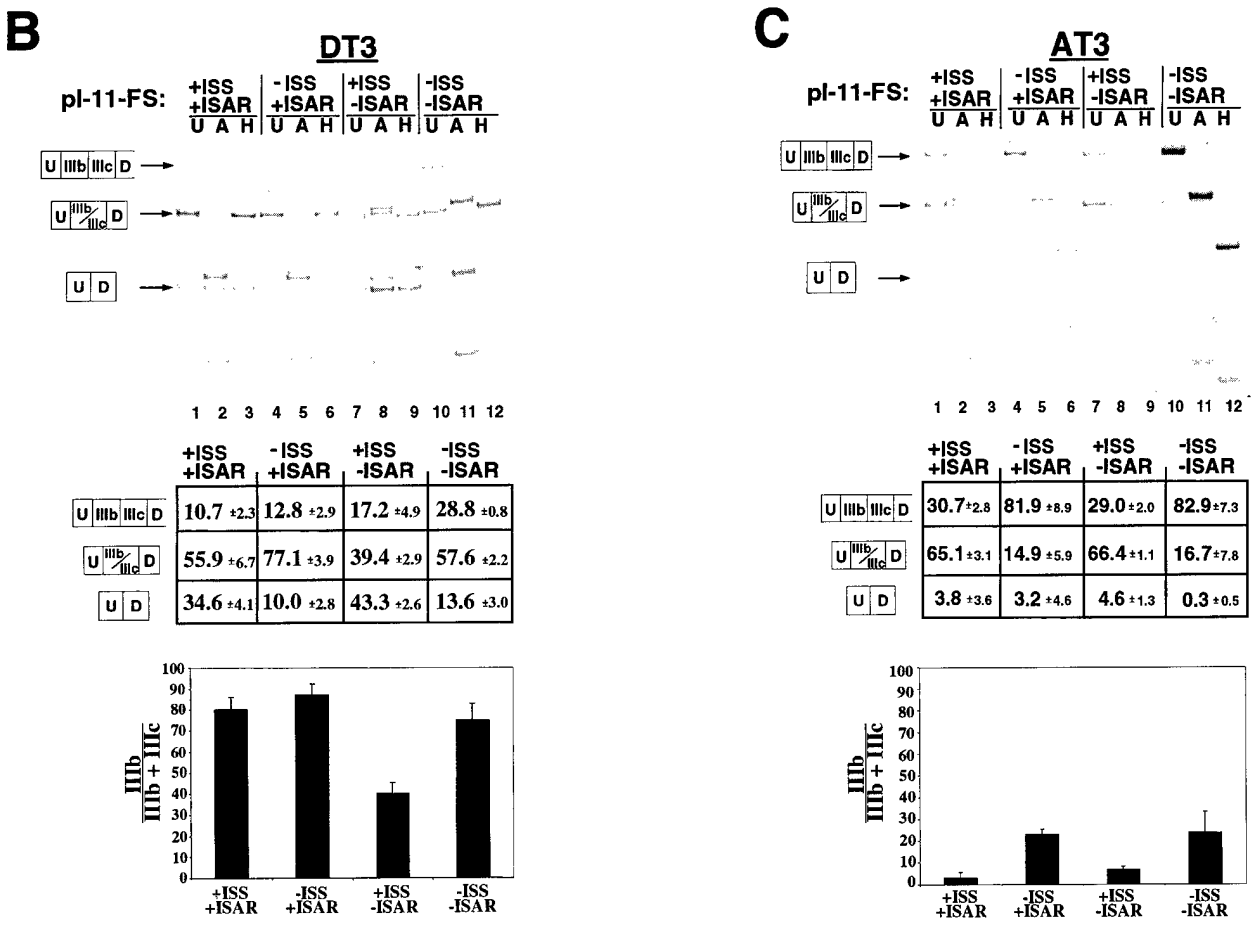
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+ISS +ISAR
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U A H
U IIIb IIIc D
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U D
1 2 3 4 5 6 7 8 9 10 11 12
10.7 ±2.3
12.8 ±2.9
17.2 ±4.9
28.8 ±0.8
55.9 ±6.7
77.1 ±3.9
39.4 ±2.9
57.6 ±2.2
34.6 ±4.1
10.0 ±2.8
43.3 ±2.6
13.6 ±3.0
IIIb / IIIb + IIIc
C
AT3
30.7±2.8
81.9 ±8.9
29.0 ±2.0
82.9±7.3
65.1 ±3.1
14.9 ±5.9
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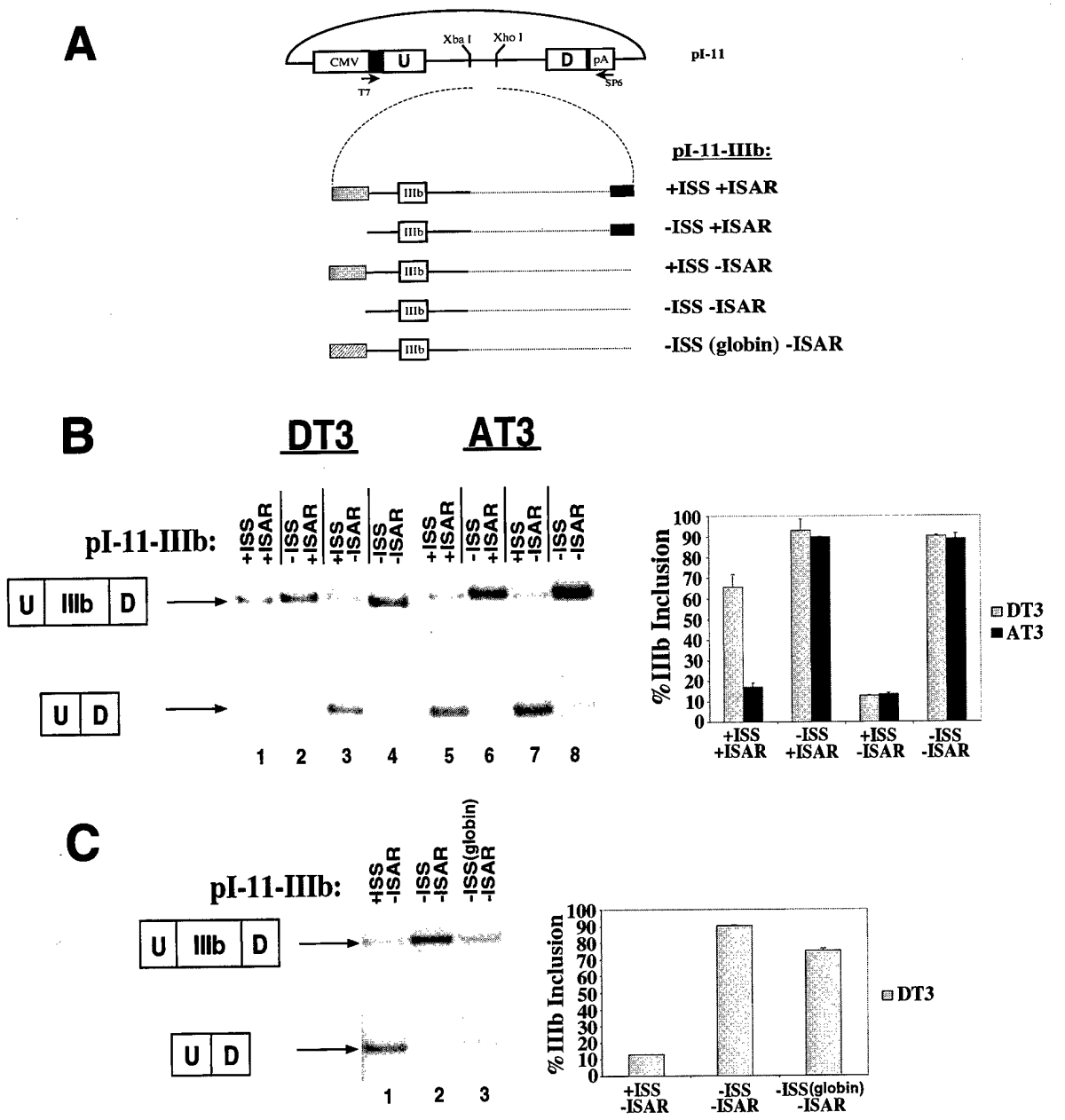

FIG. 3. Deletion of ISS increases exon IIIb inclusion in both DT3 and AT3 cell lines when tested in the absence of exon IIIc. (A) Outline of the constructs used that contain exon IIIb. Designations are the same as in Fig. 2A, except that here a hatched box is added to indicate substitution of the β-globin intron sequence. (B) The boxed figures at the left indicate the two expected products in which either IIIb is included or exon IIIb is skipped and the upstream (U) and downstream (D) adenovirus exons are spliced. Quantifications graphed in the right panel were performed as described in Materials and Methods. (C) Substitution of β-globin intron sequence has the same effect as deletion of ISS.

(1, 48, 52). Since PTB has been implicated in the repression of splicing to adjacent exons in other mammalian systems, we sought to explore whether PTB might interact with ISS1 and, further, whether this interaction might be involved in repression of exon IIIb splicing.

PTB binds to sequences within ISS1 but not to other sequences comprising the silencer element upstream of exon IIIb. To characterize specific PTB binding sequences within this region of the FGF-R2 pre-mRNA, we performed successive deletions within the RNA followed by cross-linking and immunoprecipitation using anti-PTB antibodies (data not shown). In particular, we found that PTB efficiently cross-linked to the ISS1 sequence in both DT3 and AT3 nuclear extracts (diagrammed in Fig. 5A and demonstrated in Fig. 5B, lanes 2 and 6). Immunoprecipitation of the cross-link using PTB antiserum confirmed its identity (data not shown). When the pyrimidine stretch was altered to contain a stretch of purines (ISS1mt), PTB cross-linking was greatly reduced (Fig. 5B, lanes 3 and 7). A size-matched nonspecific RNA derived from intron 2 of the human β-globin gene was also seen not to cross-link to PTB (lanes 4 and 8). All three probes cross-linked to a similar set of background bands that bound without spec-

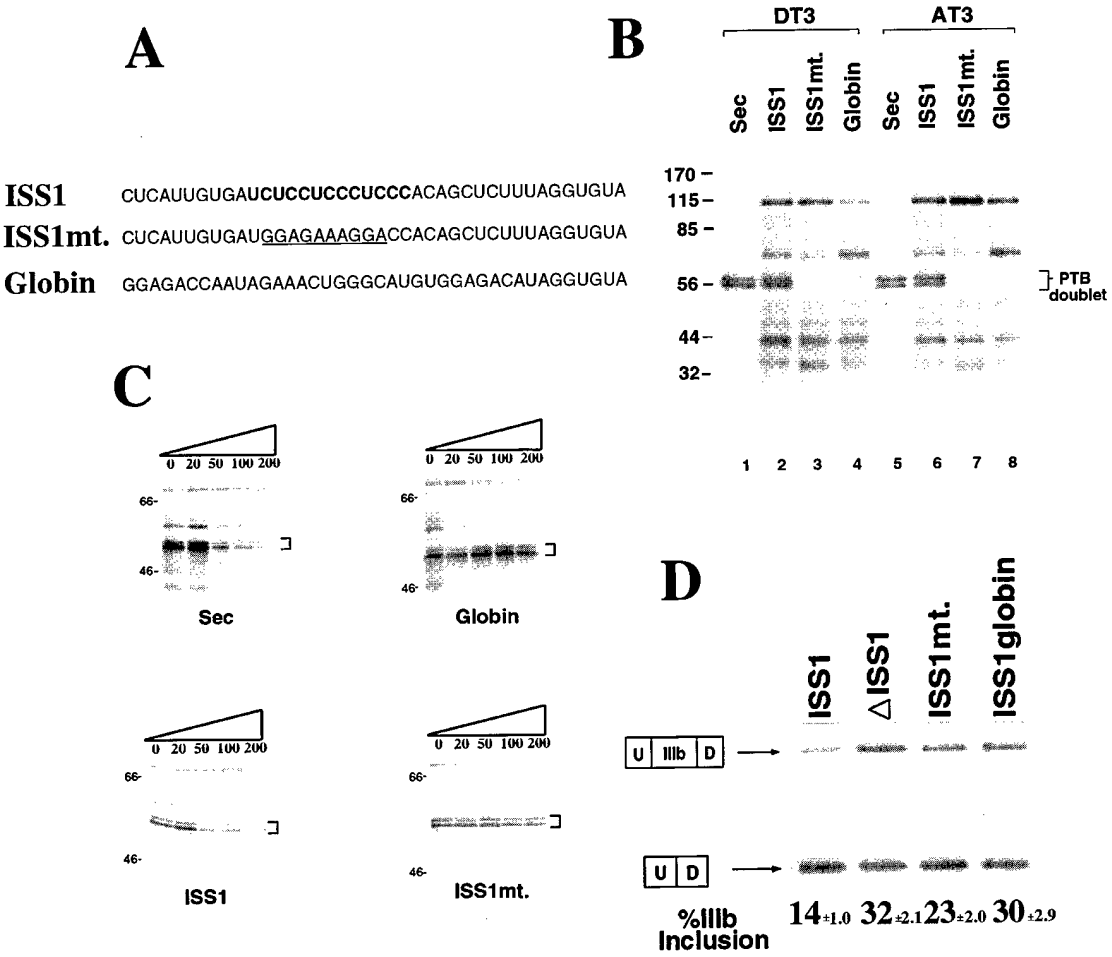

FIG. 5. ISS1 binds PTB and is required for repression of exon IIIb. (A) Diagram of the labeled RNAs used in these cross-linking studies. The pyrimidine stretch within ISS1 is highlighted in bold, while the purine mutations within that stretch in ISS1mt are underlined. The Globin sequence is an intronic sequence derived from the human β-globin gene. (B) Direct UV cross-linking of the ISS1, ISS1mt, and Globin RNAs in both DT3 and AT3 nuclear extracts. The Sec RNA is used as a positive control for PTB cross-linking. In all three cases, there are several bands common to all three RNAs; however, the PTB doublet cross-links specifically to the ISS1 and Sec RNAs, and not to the ISS mutant or substitution. The molecular weight markers (in thousands) are indicated at the left, and the cross-linked PTB bands are bracketed. (C) UV cross-linking and competition experiments demonstrated that PTB binding to the Sec RNA can be competed using self-competition or using a cold ISS1 competitor. However, the nonspecific globin competitor and ISS1mt cannot compete PTB cross-linking to the Sec RNA. For each panel, DT3 extracts were preincubated with the indicated molar fold excess of cold competitor RNA, after which the labeled Sec control probe was added prior to cross-linking. Molecular weights (in thousands) are indicated at the left, and PTB bands are bracketed. (D) Deletion, mutation, or replacement of ISS1 which abolishes PTB binding also results in the loss of exon IIIb splicing repression. These constructs were stably transfected in triplicate into DT3 cells, and the average percentage of exon IIIb inclusion is indicated.

Given that we have previously reported a change in the PTB isoform expression in DT3 and AT3 cells, we reasoned that perhaps the difference in splicing patterns could be attributed to the difference in the ability of the three PTB splice variants to repress exon IIIb inclusion. When the different PTB isoforms were overexpressed, very similar levels of exon IIIb repression were seen (Fig. 6D). This suggests that the isoforms do not differ in their repressor activity. It does not rule out, however, that the isoforms are differentially responsive to the derepression brought about by ISAR. We are currently investigating this possibility.

As a control, an equivalent amount of HisG-LacZ was transiently transfected into DT3 cells, resulting in a level of LacZ protein overexpression similar to that of PTB (Fig. 6E). There was no effect on IIIb inclusion at any level of LacZ overexpression (Fig. 6F). These experiments suggest that increasing the levels of PTB can counteract the activation effects of ISAR in DT3 cells and that this repression is dependent on the intronic splicing silencer.

DISCUSSION

In the present study, we have identified an intronic element upstream of exon IIIb in the FGF-R2 transcript which appears to exert repression, or silencing, of splicing to exon IIIb in both the DT3 and AT3 cell lines. While it is possible that cell-specific differences in the level of repression mediated by this sequence may contribute to splicing regulation, we were not able to appreciate such differences. Previous work of our laboratory as well as of others studying the human FGF-R2 gene has characterized several elements that affect the splicing of FGF-R2 exons IIIb and IIIc. An element within exon IIIb consisting of a critical UAGG sequence has been shown to inhibit the splicing of this exon and has thus been termed an

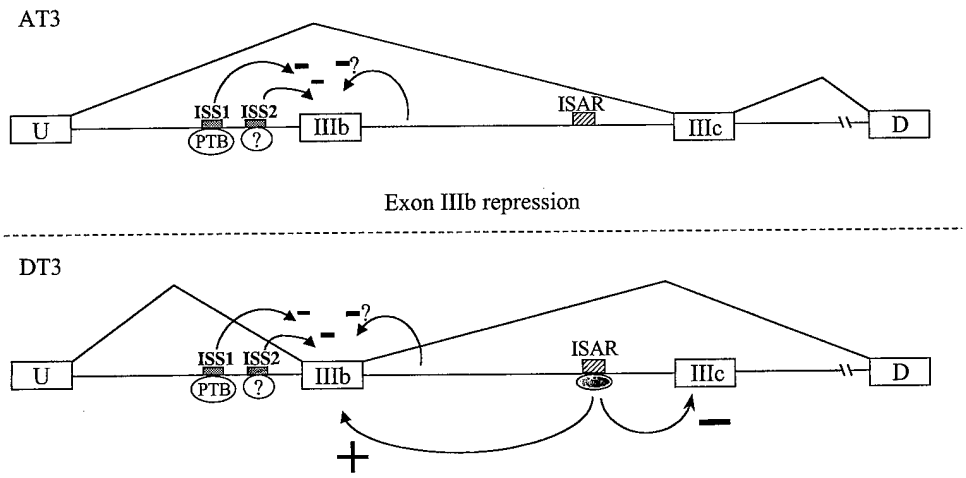

FIG. 7. Model in which a combination of positive and negative effects leads to regulation of FGF-R2 alternative splicing. We propose that AT3 cells splice IIIc efficiently and skip exon IIIb as a result of the effect of ISS that is mediated in part by PTB. In DT3 cells, the same exon IIIb splicing silencing activity is present; however, it is counteracted by exon IIIb splicing activation, which includes factors that interact with ISAR. In addition, factors that interact with ISAR contribute to the repression of splicing of exon IIIc. Shaded boxes indicate ISS1 and ISS2 elements. Involvement of PTB with ISS1 is shown. Silencing effects, which involve ISS2 as well as less well characterized sequences downstream of exon IIIb are also proposed. The location and activity of ISAR and undefined protein(s) that bind to it are also shown.

pre-mRNAs by repressing splicing in certain regions of the pre-mRNA transcript. Overexpression of PTB in vivo has been shown to promote exon inclusion of exon 4 of the CT/CGRP transcript, but these data are the first to demonstrate the repression of exon inclusion in vivo (34). In our in vivo experiments, repression was most robust when PTB was cotransfected with a minigene that contained the ISS, although some repression was still observed when this sequence was deleted. Analysis of sequences in the intron downstream of exon IIIb reveals a number of sequences, including seven UCUU motifs, which may also represent PTB binding sites (1, 48, 52). When we transfected a minigene that contained the entire ISS region and ISAR but lacked the downstream putative PTB binding sites into DT3 and AT3 cells, we noted that exon IIIb was included in at least 90% of spliced RNAs (data not shown). This high level of exon IIIb inclusion was seen both in the presence and absence of the ISS, although deletion of the ISS did result in a small increase in IIIb inclusion. These results suggest the presence of a sequence downstream of exon IIIb that can also act to silence splicing of exon IIIb. Based on these preliminary results, we suspect that the effect of PTB to decrease the efficiency of exon IIIb splicing may also involve the interaction of PTB at sequences in the downstream intron. Based on the results of other investigators, the existence of multiple PTB binding sites near an alternatively spliced exon is not unprecedented (12). Such is the case in splicing of exon 3 of α-tropomyosin and exon N1 of c-*src*, in which PTB binding to sequences both upstream and downstream of this alternative exon results in splicing repression (25, 48). Splicing repression by the Sxl protein in *Drosophila* has likewise been shown to involve interactions on both sides of a repressed exon (29). Further study is under way to characterize the elements downstream of exon IIIb, which are also likely to be involved in this repression.

In addition to the portion of ISS that binds PTB, the downstream region within this silencer, ISS2, appears to play a significant role in repressing splicing of exon IIIb. At present we have not identified any specific proteins which interact directly with this region. Analysis of the sequence would suggest that PTB does not bind to this portion of the repressor, and in fact the cross-linking competition studies have not demonstrated direct PTB binding to this region. Other hnRNP proteins are candidates for interacting with this region. Given the G-rich nature of this sequence, hnRNP H, which exhibits a preference for binding to G-rich RNA sequences, is one candidate. In fact, hnRNP H has previously been implicated in splicing repression of exon 7 of β-tropomyosin (9).

The mechanism by which PTB may function to repress splicing is at present poorly characterized. It has been suggested that PTB may prevent the binding of U2AF to the polypyrimidine tract of associated exons similar to the predicted mechanism that Sxl uses to repress the male-specific exon of the transformer pre-mRNA (52). However, this may be true only in some cases. Among the observations that question this general hypothesis are that the binding sites for Sxl and PTB are not always overlapping with the bp/py and have been shown to sometimes reside hundreds of nucleotides away (8, 29, 48). In the case of Sxl splicing autoregulation, the binding sites that are within the bp/py are less important than those located in the intron downstream of the male-specific exon (29). Furthermore, PTB binding downstream of N1 affects its ability to bind upstream, suggesting that PTB cooperativity plays a role in repression (12). There are no proteins that interact with PTB that have also been demonstrated to be functionally relevant for its role in alternative splicing. However, several proteins, including the FUSE-binding protein and the Sam68 tyrosine phosphoprotein, have been shown to exist in a complex with PTB assembled on a PTB binding sequence upstream of the repressed exon 7 of β-tropomyosin (26). Furthermore, like Sxl, PTB has been shown to interact with itself and to exist as a dimer in solution, raising the possibility that the dimerization of these proteins is a necessary event for interaction with other potential proteins and their repression of exon inclusion (49, 58). This is a logical possibility, given the existence of PTB or Sxl binding sites clustered on either side of certain regulated exons.

As a group, hnRNP proteins are known to rapidly associate with hnRNAs cotranscriptionally, and, in addition to packag-

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Fluorescent Reporters to Recapitulate *in vivo* Changes in FGF-R2 Alternative Splicing

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Fibroblast growth factor receptor 2 (FGF-R2) alternative splicing occurs in a highly tissue restrictive fashion. The mutually exclusive inclusion of exon IIIb or IIIc results in receptors with different ligand binding specificities. The general profile of FGF-R2 splicing demonstrates that the IIIb exon is predominantly included in epithelial cells while the IIIc exon is included in mesenchymal tissues. In recent elegant experiments, either the IIIb or IIIc exon has been knocked out in mice. FGF-R2-IIIb$^{-/-}$ mice survive to term but show severe dysgenesis and agenesis of many internal organs. Specific knockout of FGF-R2-IIIc results in an embryonic lethality, however, hemizygous knockouts demonstrate many of the physical abnormalities associated with Apert's and Pfeiffer's syndromes.

Our lab has been studying the mechanisms of FGF-R2 alternative splicing using two rat cell lines that splice the endogenous FGF-R2 either to the IIIb exon or the IIIc exon, exclusively. Using this system we have identified several *cis* regulatory elements. In AT3 cells, which include IIIc, we have found that two Intronic Splicing Silencers (ISS) flank the IIIb exon and are each required for its repression. In DT3 cells, two cell-type specific *cis* elements, termed the Intronic Activating Sequence 2 (IAS2) and the Intronic Splicing Activator and Repressor (ISAR) have been identified to overwhelm the repressive effect of the flanking ISS. These two elements can form a putative secondary structure that we believe physically interferes with the activity of downstream ISS due to its proximity with IAS2. Our research on the mechanism of FGF-R2 alternative splicing has focused primarily on *in vitro* systems and tissue culture cell lines. We are interested in studying the physiological relevance of these mechanisms within the living animal to gain insight both on the developmental regulation of FGF-R2 alternative splicing as well as the regulation within the adult mouse.

We are developing a versatile set of fluorescent reporters using both GFP and RFP. Within the GFP reading frame we have introduced FGF-R2 exon IIIb as well as its regulatory *cis* elements. Inclusion of the IIIb exon within GFP cDNA results in an insertion, which disrupts fluorescence; only cells that skip the exon will fluoresce. Using this reporter, we have been able to demonstrate striking cell-type specific GFP fluorescence. We are also introducing the IIIc exon as well as its *cis* element regulatory sequences into the RFP reading frame. We are performing experiments aimed at illustrating cell-type specific fluorescence of RFP. These reporters, when used in concert, act as complementary measurements of activity of the splicing factors responsible for tissue-specific FGF-R2 alternative splicing. We intend to express these reporters in mice using tissue constitutive promoters to follows patterns of tissue specific FGF-R2 splicing.

We have recently shown that the Polypyrimidine Tract Binding Protein (PTB) is involved in mediating silencing of IIIb through its interaction with the both the ISS elements. A GFP reporter containing IIIb and the flanking ISS has been designed to fluoresce in the presence of PTB activity. We will demonstrate these findings as well as discuss current experiments aimed at testing the reporter's ability to demonstrate PTB activity within a variety of cell lines containing variable levels of PTB. The ultimate goal of this reporter is to generate a mouse capable of reporting PTB activity within tissue types by virtue of the GFP fluorescence.

Cell-type specific inclusion of FGF-R2 exon IIIb is mediated by a central switch: the intronic control element.

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Tissue specific alternative splicing of fibroblast growth factor receptor 2 (FGF-R2) pre-mRNA results in the mutually exclusive use of exons IIIb or IIIc; choice of exon inclusion determines the ligand specificity and has profound physiological consequences. The well-differentiated DT3 rat prostate cell line includes IIIb, while the poorly differentiated AT3 rat prostate line includes IIIc. We will present experiments directed at unraveling the cell-type specific use of IIIb or IIIc as well as a system designed for the study of FGF-R2 splicing in living cells or transgenic animals.

In both DT3 and AT3 cells, two intronic splicing silencers (ISS) located upstream of exon IIIb (UISS) and downstream (DISS) promote its exclusion. Both ISS have multiple binding sites for the Polypyrimidine Tract Binding Protein (PTB) and PTB is a major player in mediating silencing of IIIb via the UISS. Although silencing via the DISS also involves PTB; analysis revealed a critical requirement for other factors that bind a conserved UGCUU motif, this will be discussed. We believe the ISS elements, in large measure via PTB, form the borders of a zone of silencing; IIIb sits in the middle of this zone. In DT3 cells, however, the ISS are countered by two cis-acting elements located between IIIb and IIIc; these elements working in concert activate IIIb, and independently repress IIIc. These intronic splicing activators and repressors (ISAR and ISAR2), which have no apparent function in AT3 cells, contain complementary sequences and can form a stem (as proposed by R. Breathnach). ISAR2 is embedded within the DISS; given this arrangement it's likely that an ISAR-ISAR2 stem will disrupt ISS function. The DISS elements and the overlapping ISAR2 form a master switch for cell-type specific regulation of IIIb inclusion; we have named this switch: intronic control element (ICE).

In order to study the interplay between the ISS and ISAR elements during development or tumor progression we have developed a system that relies on the expression of Green Fluorescent Protein. We will show the system to be a versatile and accurate reporter of ISS and ISAR function. We will also discuss applications of the system to study alternative splicing in transgenic animals and in tumors in situ.